Automated Separation of Crude Extracts from Natural Products into Fractions for Primary High Throughput Screening

Application Note PHA0312

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Keywords

Gilson 215 Liquid Chromatography System, TRILUTION® LC Liquid Chromatography Software, high Throughput Screening, and Natural Products.

Introduction

The screening of natural products (NP) is an important component of the drug discovery process with approximately 50% of approved drugs being derived from NPs (1). These NPs come from complex biological systems which, if not removed, can produce problems that affect screening assays. This application note describes an automated prefractionation process, where crude extracts of microbial cultures are fractionated prior to primary screening. The automated prefractionation process increased assay hit rates without a reduction of hit quality (2).

Benefits

- Concentrates metabolites by removing salts, sugars, lipids etc
- Minor active compounds more likely to be discovered
- Compounds with activity masked in the crude extract more likely to be discovered
- Fractions can be selectively screened to avoid interference compounds
- Crude extracts screened alongside fractions
- Fractions tested at equivalent dose at >10 times crude concentration

Materials & Methods

Materials

- Microbial extracts
- Methanol
- Automated LC-RP C18 system
    - Gilson 215 with 322 Gradient Mobile Phase Pump and 155 Dual Wavelength Detector (Figure 1)
    - Mobile Phase
        - A: Milli-Q H2O with 0.1% formic acid
        - B: Acetonitrile with 0.1% formic acid
    - Waters Xterra® RP18 30x19mm column
    - Waters Xterra RP18 Guard column 10 mm
- Genevac HT-8 centrifugal evaporator

Method

- 48 samples prepared for overnight automated fractionation
- 1.4 mL methanol added to approximately 500 mg dried microbial extracts
- Samples sonicated for 1 hour
- Transfer extract to 96 well plate and centrifuge
- 96 well plate placed onto Gilson 215 bed (Figure 2)
- 1 mL (400-500 mg) sample injected onto C18 column
    - Flow rate: 9 mL/min
    - 8 minute gradient (0-100% B)
- Fractions collected into custom racks holding Genevac evaporator racks
    - 16 x 100 mm tubes
    - Start at the tail end of the solvent front
    - Fractions (4) collected every 1.44 minutes (4 fractions, 12 mL total volume)
- Fractions dried using the Genevac HT-8 centrifugal evaporator (12 hour cycle)
- Dried fractions reconstituted in methanol and split into 5 assay plates for screening

Figure 1. Gilson 215 with 322 Gradient Mobile Phase Pump and 155 Dual Wavelength Detector

Figure 2. Automated LC-RP C18 system; A) Crude Extracts, B) Gilson 215 System with Custom Racks Holding Genevac Evaporator Racks, C) C18 Reverse Phase Column with Guard.

Results

The majority (80%) of the crude extract mass was eluted off the column with the solvent front and therefore not collected (Figure 3). The late eluting lipophilic compounds were also not collected. The prefractionation of crude extract allowed major components to be screened separately and minor components to be separated from other interfering chemicals and concentrated to determine activity.

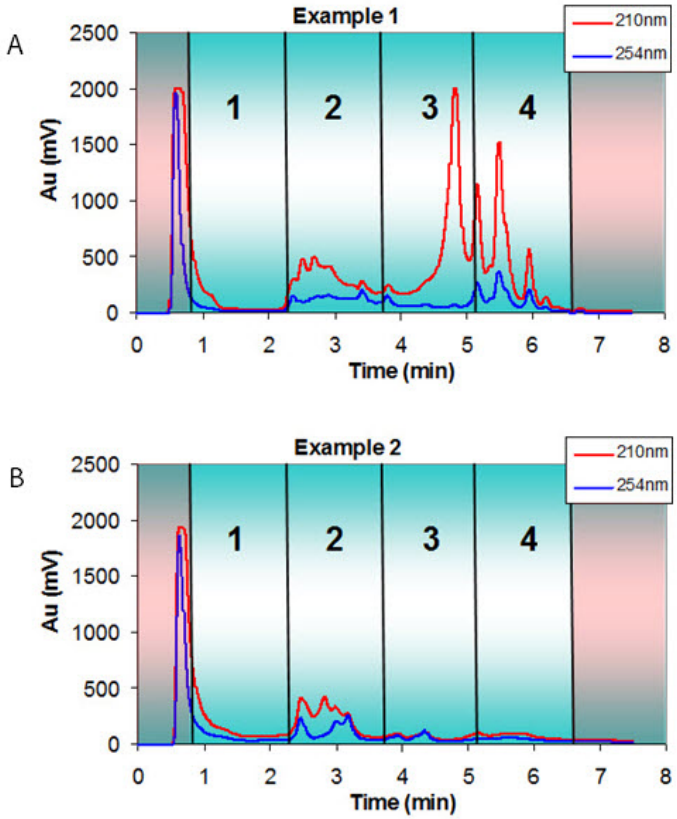

Figure 3. Example Chromatogram of Prefractionated Microbial Crude Extract. Four Fractions Collected Following Solvent Front. A) Major Components in Fraction 3 and 4 can be Divided into Fractions and Screened Separately. B) Minor Components in Fractions 3 and 4 can be Concentrated and Separated From (media) Components in Fraction 2.

Crude extracts and fractions were screened for biological activity such as antimicrobial %inhibition. The crude extract and fractions of sample T467 were not active at equivalent doses; however fraction 3 was active following prefractionation (Figure 4). The active compounds in fraction 3 were minor compounds (0.3% wt/wt) with weak activity (30-50 µM), which was masked or blocked in the crude extract. The crude extract of sample T174 showed no activity, while the equivalent dose of fraction 3 did (Figure 5). Compounds in the solvent front of T174 masked the effects of fraction 3 despite good activity (1 µM) and high concentration (1% wt/wt) of the compound of interest.

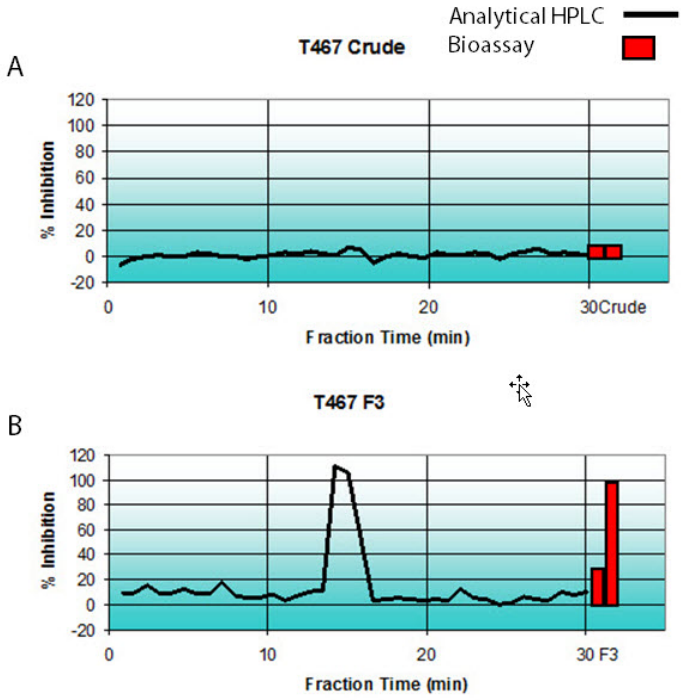

Figure 4. Equivalent Dose LC-Bioassay Plots of a Sample Demonstrating How Minor Compounds are Concentrated by Prefractionation by Comparing A) Inactive T467 Crude Extract and B) Active T467 Fraction 3.

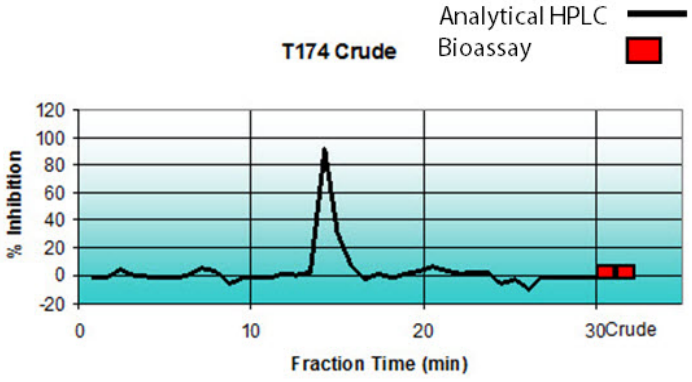

Figure 5. Equivalent Dose LC-Bioassay Plots for T174 Crude.

Summary

An analysis of 1700 hits from 11 screens showed that 80% of the total number of hits came from fractions alone following prefractionation and less than 10% of were found in the crude extract alone (Figure 6). Most hits (84%) were only found in a single fraction, with fractions 2-4 getting reasonable distribution (Figure 7). There were over 12 times more hits found in the fractions than in the crude alone. The hit rate per extract was 5 times higher following prefractionation, with a 20% increase in compound novelty. Compound activities (IC50) following prefractionation remained similar or better than previous methods. These data show the benefits of prefractionation of crude extract prior to screening.

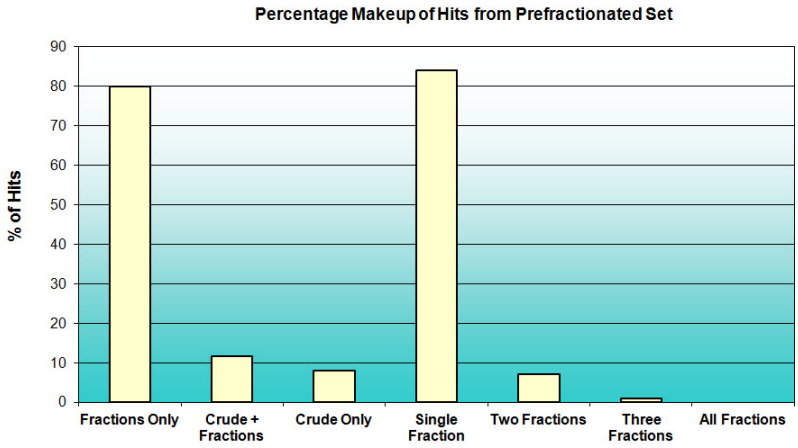

Figure 6. Percentage Makeup of Hits From Prefractionated Set. The Majority of Hits Came From the Fractions Only, With 84% of Hits Being Found in a Single Fraction.

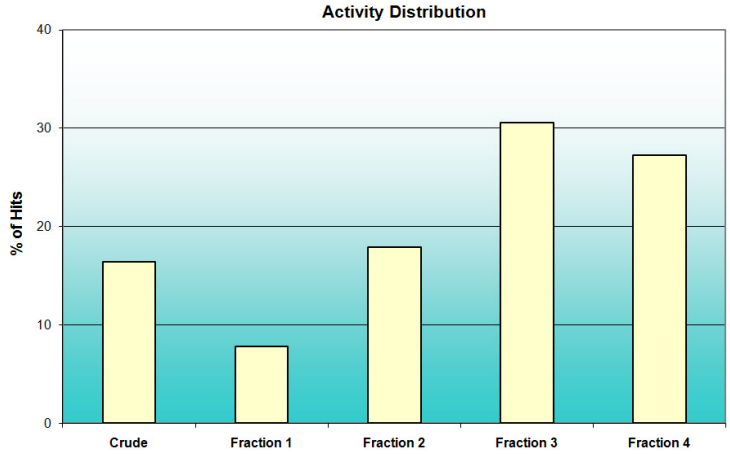

Figure 7. Activity Distribution. While the Majority of Hits Came From Fractions 2-4, There is Fairly Even DistributionAcross These 3 Fractions.

References

1. Newman DJ, and Cragg GM. Natural Products as Sources of New Drugs over the Last 25 Years. J. Nat. Prod. 2007; 70:461-477
2. Appleton DR, Buss AD, Butler MS. A Simple Method for High-Throughput Extract Prefractionation for Biological Screening. Chimia. 2007; 61:327‑331